

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C07C 323/60, C07K 7/16, 14/62, C07C 323/59, 323/54, C07D 307/34, A61K 31/195, 38/11, 38/28, 47/48	A1	(11) International Publication Number: WO 00/34236 (43) International Publication Date: 15 June 2000 (15.06.00)
(21) International Application Number: PCT/US99/29119 (22) International Filing Date: 9 December 1999 (09.12.99) (30) Priority Data: 60/111,784 10 December 1998 (10.12.98) US (71) Applicant: UNIVERSITY OF SOUTHERN CALIFORNIA [US/US]; 3716 South Hope Street, Suite 313, Los Angeles, CA 90007 (US). (72) Inventors: SHEN, Wei-Chiang; 2030 Robin Road, San Marino, CA 91108 (US). HEIATI, Hashem; 13318 Huston Street, Sherman Oaks, CA 91423 (US). (74) Agents: ESMOND, Robert, W. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).	(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: REVERSIBLE AQUEOUS pH SENSITIVE LIPIDIZING REAGENTS, COMPOSITIONS AND METHODS OF USE (57) Abstract The present invention provides lipidized conjugates comprising an amino group-containing biologically active substance and a lipophilic group capable of penetrating a biological membrane. Under neutral or mildly acidic conditions, including those found <i>in vivo</i> , the free amino group-containing biologically active substance is released from the conjugate by hydrolysis of an amide bond. The present invention is also directed to methods of preparing lipidizing agents and lipidized conjugates, pharmaceutical compositions comprising lipidized conjugates and methods of increasing the delivery of amino group-containing substances into a cell. Preferred amino group-containing substances include peptides, proteins and derivatives thereof.		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

Reversible Aqueous pH Sensitive Lipidizing Reagents, Compositions and Methods of Use

Background of the Invention

Field of the Invention

5 The present invention relates generally to the fields of biology and medicine. More particularly, the present invention is directed to compounds, methods and compositions useful in increasing in mammals the transport and delivery of hydrophilic molecules having an amino group, in particular peptides and proteins.

10 *Related Art*

Advances in biochemistry have made possible the production of large amounts of therapeutically active and pure proteins and peptides. Currently, the therapeutic effects of most of these agents can be achieved only when they are administered via invasive routes, such as by injection. Since most proteins have
15 very short half lives, effective concentrations of these agents can be maintained only when administered by frequent injections.

Although the administration of protein by injection is the most effective means of their delivery *in vivo*, patient tolerance of multiple injections is very poor. In addition, drug injection requires training and skill that may not always
20 be transferable to patients. In cases where protein drugs have a life-saving role, the administration by injection can be acceptable by the patients. However, in cases where protein drugs are just one of several possible therapies, injections of proteins and peptides are unlikely to be accepted by the patients. Therefore, alternative routes of protein and peptide delivery need to be developed.

25 Such alternative routes may include the buccal, nasal, oral, pulmonary, rectal and ocular routes. Without exception, these routes are less effective than the parenteral routes of administration, but are still far more attractive than the parenteral routes because they offer convenience and control to the patients. The

oral route is particularly attractive because it is the most convenient and patient-compliant.

Mucosal barriers, which separate the inside of the body from the outside (e.g., gastrointestinal, ocular, pulmonary, rectal and nasal mucosa), comprise a layer of tightly joined cell monolayers which strictly regulate the transport of molecules. Individual cells in barriers are joined by tight junctions which regulate entry into the intercellular space. Hence, the mucosa is at the first level a physical barrier, transport through which depends on either the transcellular or the paracellular pathways (Lee, V.H.L., *Critical Rev. Ther. Drug Delivery Sys.* 5:69-97 (1988)).

Paracellular transport through water filled tight junctions is restricted to small molecules (MW < 1 kDa) and is essentially a diffusion process driven by a concentration gradient across the mucosa (Lee, V.H.L., *Critical Rev. Ther. Drug Delivery Sys.* 5:69-97 (1988); Artursson, P. and Magnusson, C., *J. Pharm. Sci.* 79:595-600 (1990)). The tight junctions comprise less than 0.5% of the total surface area of the mucosa (Gonzalez-Mariscal, L.M., *et al.*, *J. Membrane Biol.* 86:113-125 (1985); Vetvicka, V. and Lubor, F., *Critical Rev. Ther. Drug Deliv. Sys.* 5:141-170 (1988)); therefore, they play only a minor role in the transport of protein drugs across the mucosa.

The transcellular transport of small drugs occurs efficiently provided the physicochemical properties of the drug are suited to transport across hydrophobic cell barriers. However, the transcellular transport of proteins and peptides is restricted to the process of transcytosis (Shen, W.C., *et al.*, *Adv. Drug. Deliv. Rev.* 8:93-113 (1992)). Transcytosis is a complex process in which proteins and peptides are taken up into vesicles from one side of a cell, and are subsequently shuttled through the cell to the other side of the cell, where they are discharged from the endocytic vesicles (Mostov, K.E. and Semister, N.E., *Cell* 43:389-390 (1985)). The cell membrane of mucosa barriers is a hydrophobic lipid bilayer which has no affinity for hydrophilic, charged macromolecules like proteins and peptides. In addition, mucosa cells may secrete mucin which can act as a barrier to the transport of many macromolecules (Edwards, P., *British Med. Bull.* 34:55-

56 (1978)). Therefore, unless specific transport mechanisms exist for proteins and peptides, their inherent transport across mucosa barriers is almost negligible.

In addition to providing a tight physical barrier to the transport of proteins and peptides, mucosa barriers possesses enzymes which can degrade proteins and peptides before, after, and during their passage across the mucosa. This barrier is referred to as the enzymatic barrier. The enzymatic barrier consists of endo- and exopeptidase enzymes which cleave proteins and peptides at their terminals or within their structure. Enzymatic activity of several mucosa have been studied and the results demonstrated that substantial protease activity exists in the homogenate of buccal, nasal, rectal and vaginal mucosa of albino rabbits and that these activities are comparable to those present in the ilium (Lee, V.H.L., *Critical Rev. Ther. Drug Delivery Sys.* 5:69-97 (1988)). Therefore, regardless of the mucosa being considered, the enzymatic barrier present will feature strongly in the degradation of the protein and peptide molecules.

The N and the C termini of peptides are charged and the presence of charged side chains imparts highly hydrophilic characteristics on these macromolecules. In addition, the presence of charged side chains means that proteins and peptides have strong hydrogen bonding capacities; this H-bonding capacity has been demonstrated to play a major role in inhibiting the transport of even small peptides across cell membranes (Conradi, R.A., *et al.*, *Pharm. Res.* 8:1453-1460 (1991)). Therefore, the size and the hydrophilic nature of proteins and peptides combine to severely restrict their transport across mucosa barriers.

One approach that has been used to alter the physical nature of the mucosa barriers is the use of penetration enhancers. The use of penetration enhancers is based on the disruption of the cell barriers by low molecular weight agents which can fluidize cell membranes (Kaji, H., *et al.*, *Life Sci.* 37:523-530 (1985)), open tight junctions (Inagaki, M., *et al.*, *Rhinology* 23:213-221 (1985)), and create pores in the cell membrane (Gordon, S., *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7419-7423 (1985); Lee, V.H.L., *et al.*, *Crit. Rev. Ther. Drug. Carrier Syst.* 8:91-192 (1991)). The use of these agents leads to a non-specific loss of barrier

integrity and can lead to the absorption of a variety of large molecules which can be toxic to cells *in vivo*.

5 Protease inhibitors have been co-administered with proteins and peptides and have shown some limited activity in enhancing the absorption of these macromolecules *in vivo* (Kidron, M., *et al.*, *Life Sci.* 31:2837-2841 (1982); Takaroi, K., *et al.*, *Biochem. Biophys. Res. Comm.* 137:682-687 (1986)). The safety and the long-term effects of this approach have yet to be thoroughly investigated.

10 The prodrug approach is based on the modification of peptides in a manner that will protect them from enzyme degradation and recognition. This has been achieved by the blockage of vulnerable groups on peptides by amidation and acylation. The prodrug approach has thus far proven useful only for small peptides which have easily identifiable domains of activity.

15 Reduction in size is another feasible approach to increasing the transport potential of proteins. However, the active sites of proteins need to be mapped before size reduction can be attempted. In general, this approach is difficult to apply to the majority of proteins.

20 Carrier ligands, by virtue of their properties, can alter the cell uptake and transport characteristics of proteins and peptides. The essence of this approach is that a cell-impermeant protein or peptide is covalently attached to a carrier which is highly transported into cells. The mechanisms through which carrier ligands became endocytosed and transcytosed are important in deciding the suitability of the carrier for enhancing the transport of proteins and peptides. Macromolecular carriers are hydrophilic and do not partition into the membrane. 25 Therefore, the transport of large polymeric carriers into the cells is mediated by the affinity of the carrier for the cell membrane. Generally, the uptake of a macromolecular conjugate starts with binding to the cell membrane. The binding of the carrier to the cells can be specific (e.g., binding of antibodies to cell surface antigens), nonspecific (binding of cationic ligand or lectins to cell surface sugars), 30 or receptor mediated (binding of transferring or insulin to their receptors). Once

the carrier is bound to the cell surface, it is taken up into vesicles. These vesicles then become processed stepwise and can be routed to several pathways. One pathway is the recycling of the vesicle back to the membrane. Another pathway, which is destructive to the conjugate, is the fusion with lysosomes. An alternative pathway, and one which leads to the transcytosis of the conjugate, is the fusion of the vesicle with the membrane opposite to the side from which it was derived.

The correct balance between the processes of endocytosis and transcytosis determine the delivery of a protein conjugate to its target. For instance, endocytosis may determine the extent to which a conjugate is taken up by the target cell, but transcytosis determines whether or not a conjugate reaches its target (Shen, W.C., *et al.*, *Adv. Drug. Deliv. Rev.* 8:93-113 (1992)). For successful absorption through the gastrointestinal tract, a conjugate must bind the apical membrane of the gastrointestinal mucosa, become internalized into the mucosa cells, be delivered across the cells, and finally become released from the basolateral membrane.

The current literature contains many reports which demonstrate that nonspecific carriers, such as polylysines (Shen, W.C. and Ryser, H.J.P., *Proc. Natl. Acad. Sci. USA* 78:7589-7593 (1981)) and lectins (Broadwell, R.D., *et al.*, *Proc. Natl. Acad. Sci. USA* 85:632-646 (1988)), and specific carriers, such as transferrin (Wan, J., *et al.*, *J. Biol. Chem.* 257:13446-13450 (1992)), asialoglycoprotein (Seth, R., *et al.*, *J. Infect. Diseases* 168:994-999 (1993)), and antibodies (Vitetta, E.S., *J. Clin. Immunol.* 10:15S-18S (1990)) can enhance the endocytosis of proteins into cells. Reports dealing with transcytotic carriers for proteins are fewer, and very few studies have quantitated the transport of protein conjugates across cell barriers. Wheat germ agglutinin (Broadwell, R.D., *et al.*, *Proc. Natl. Acad. Sci. USA* 85:632-646 (1988)) and an anti-transferrin/methotrexate conjugate (Friden, P.M. and Walus, L.R., *Adv. Exp. Med. Biol.* 331:129-136 (1993)) have been shown to be transcytosed across the blood-brain barrier *in vivo*. Also, polylysine conjugates of horseradish peroxidase

(HRP) and a transferrin conjugate of HRP have been shown to be transcytosed across cell monolayers *in vitro* (Wan, J. and Shen, W.C., *Pharm. Res.* 8:S-5 (1991); Taub, M.E. and Shen, W.C., *J. Cell. Physiol.* 150:283-290 (1992); Wan, J., *et al.*, *Biol. Chem* 267:13446-13450 (1992)).

5 Fatty acids, as constituents of phospholipids, make up the bulk of cell membranes. They are available commercially and are relatively cheap. Due to their lipidic nature, fatty acids can easily partition into and interact with the cell membrane in a non-toxic way. Therefore, fatty acids represent potentially the most useful carrier ligand for the delivery of proteins and peptides. Strategies that may use fatty acids in the delivery of proteins and peptides include the covalent modification of proteins and peptides and the use of fatty acid emulsions.

10 Some studies have reported the successful use of fatty acid emulsions to deliver peptide and proteins *in vivo* (Yoshikawa, H., *et al.*, *Pharm. Res.* 2:249-251 (1985); Fix, J.A., *et al.*, *Am. J. Physiol.* 251:G332-G340 (1986)). The mechanism through which fatty acid emulsions influence the absorption of proteins and peptides is not yet known. Fatty acid emulsions may open tight junctions, solubilize membranes, disguise the proteins and peptides from the gastrointestinal environment, and carry proteins and peptides across the gastrointestinal mucosa as part of their absorption (Smith, P., *et al.*, *Adv. Drug. Delivery Rev.* 8:253-290 (1992)). The latter mechanism has been proposed, but is inconsistent with current knowledge about the mechanism of fat absorption.

20 A more logical strategy to deliver proteins and peptides across the gastrointestinal epithelium is to make use of fatty acids as non-specific membrane adsorbing agents. Several studies have shown that a non-specific membrane binding agent linked to a protein can promote the transcytosis of a protein conjugate across cells *in vitro* (Wan, J., *et al.*, *J. Cell. Physiol.* 145:9-15 (1990); Taub, M.E. and Shen, W.C., *J. Cell. Physiol.* 150:283-290 (1992)). Fatty acid conjugation has also been demonstrated to improve the uptake of macromolecules into and across cell membranes (Letsinger, R., *et al.*, *Proc. Natl. Acad. Sci. USA*

25

30

86:6553-6556 (1989); Kabanov, A., *et al.*, *Protein Eng.* 3:39-42 (1989)).
Nonetheless, there have been difficulties in conjugating fatty acids to peptides
and proteins, including: (1) the lack of solubility of fatty acids in the aqueous
solution for the conjugation reaction; (2) the loss of biological activity of peptides
5 and proteins after fatty acid acylation; and (3) the lack of solubility of fatty acid-
conjugated peptides in aqueous solutions (see, e.g., Hashimoto, M., *et al.*, *Pharm.*
Res. 6:171-176 (1989); Martins, M.B.F., *et al.*, *Biochimie* 72:671-675 (1990);
Muranishi, S., *et al.*, *Pharm. Res.* 6:171-176 (1989); Martins, M.B.F., *et al.*,
Biochimie 72:671-675 (1990); Muranishi, S., *et al.*, *Pharm. Res.* 8:649-652
10 (1991); Robert, S., *et al.*, *Biochem. Biophys. Res. Commun.* 196:447-454 (1993)).

Once delivered into the cell, peptides and proteins must be released from
their carrier. Published PCT Application Nos. WO 96/22773 and WO 98/13007
disclose the transcellular delivery and release of sulfhydryl-containing peptides
and proteins. The cellular absorption of sulfhydryl-containing hydrophilic
15 molecules can be increased by conjugation with a fatty acid through a disulfide
linkage. The labile disulfide linkage is easily reduced, providing a mechanism
for the release of the hydrophilic compounds from the fatty acid moiety once
inside the body.

In addition to disulfide bond reduction, other mechanisms for the release
20 of biologically active hydrophilic compounds from carrier systems include
hydrolysis and photolytic bond cleavage. (See for example, U.S. Pat. No.
5,505,931 and references cited therein). Hydrolysis-based delivery systems in
which a biologically active amine is conjugated with an organic acid
incorporating a monoclonal antibody or other substrate for the targeting of
25 specific cells are known. (See U.S. Pat. Nos. 4,764,368, 4,618,492, 5,505,931
and 5,563,250). After specific binding to the targeted cell, these conjugates
deliver the active amine (typically in the form of an amide) inside the cell where
hydrolysis (of the amide) releases the free amine inside the cell.

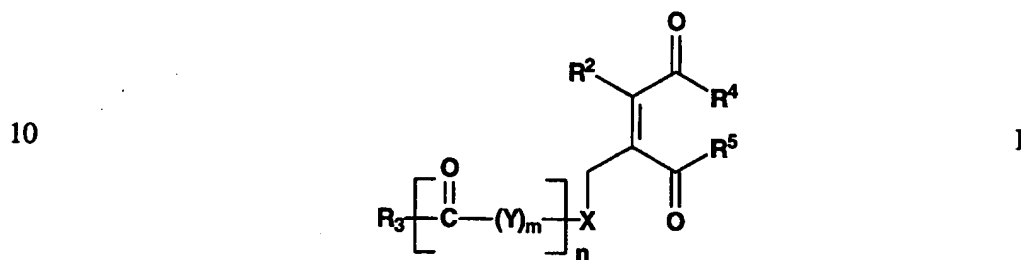
The success of prior art hydrolysis-based delivery systems has inspired the
30 search for improved drug-carrier conjugates capable of delivering a biologically

active amino group containing compound to the inside of cells. Improved synthetic strategies and treatment techniques are currently being developed.

Summary of the Invention

5 The present invention relates to new drug-carrier conjugates and convenient synthetic strategies for their production. Accordingly, the present invention is directed to synthetic methods, intermediates and ultimately final products useful for the uptake and release of biologically-active amino group containing compounds.

In particular, the invention relates to compounds of general Formula I



in which R² is selected from the group consisting of hydrogen, halo, alkyl, or aryl, wherein the alkyl or aryl groups are optionally substituted with one or more alkoxy, alkoxyalkyl, alkanoyl, nitro, cycloalkyl, alkenyl, alkynyl, alkanoyloxy, alkyl or halogen atoms;

15 R³ is a lipophilic group;

one of R⁴ and R⁵ is a biologically active amino group containing substance selected from the group consisting of an amine-containing drug, a natural or unnatural amino acid, a peptide and a protein and the other of R⁴ and R⁵ is OR⁶ where R⁶ is hydrogen, an alkali metal or a negative charge;

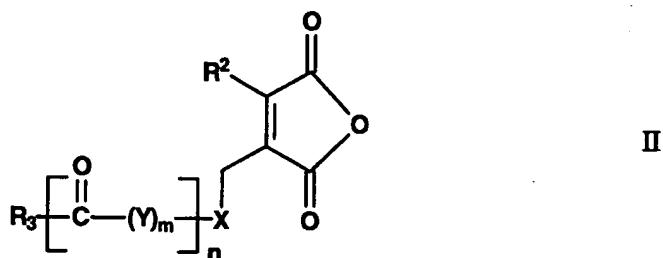
20 X is oxygen or sulfur;

Y is a bridging natural or unnatural amino acid;

n is zero or 1; and

m is an integer from zero to 10.

The present invention also relates to compounds of the general Formula II



in which R² is hydrogen, halo, alkyl, or aryl, wherein the alkyl and aryl groups are optionally substituted with one or more alkoxy, alkoxyalkyl, alkanoyl, nitro, cycloalkyl, alkenyl, alkynyl, alkanoyloxy, alkyl or halogen atoms;

R³ is a lipophilic group;

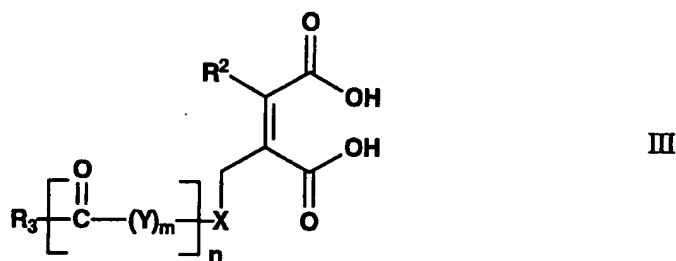
X is O or S;

Y is a bridging natural or unnatural amino acid;

n is zero or 1; and

m is an integer from zero to 10.

The present invention also relates to compounds of the general Formula III



or a pharmaceutically-acceptable salt thereof,

in which R² is hydrogen, halo, alkyl, or aryl, wherein the alkyl and aryl groups are optionally substituted with one or more alkoxy, alkoxyalkyl, alkanoyl, nitro, cycloalkyl, alkenyl, alkynyl, alkanoyloxy, alkyl or halogen atoms;

R³ is a lipophilic group;

X is O or S;

Y is a bridging natural or unnatural amino acid;

n is zero or 1; and

m is an integer from zero to 10.

5 The present invention also relates to methods of forming conjugates of general Formula I from compounds of general Formula II and a biologically active amino group containing substance.

The present invention also relates to methods of forming compounds of general Formula II from maleic acid derivatives and the corresponding thiols or alcohols.

10 The present invention also relates to methods for increasing the absorption or prolonging blood and tissue retention in a mammal of a biologically active amino group containing substance, in which a conjugate of general Formula I is administered to the mammal in a pharmaceutically-acceptable form.

15 The present invention also relates to methods for increasing the delivery of hydrophilic amine containing compounds to the inside of a cell having a mucosal barrier, in which a conjugate of general Formula I is contacted with the cell whereby the conjugate penetrates the mucosal barrier of the cell and the free amine is liberated by hydrolysis of an amide bond.

The present invention also relates to pharmaceutical compositions comprising a compound of general Formula I.

20 The above and other features, advantages, embodiments, aspects and objects of the present invention will be clear to those skilled in the areas of relevant art, based upon the description, teaching and guidance presented herein.

Brief Description of the Drawings

25 FIG. 1 shows the pH dependence of the release of tyramine from a lipidization carrier reagent (REAL-tyramine) in accordance with the invention. The data show the mean and SD of 3 experiments.

FIG. 2 shows the cumulative urine output of diabetic rats after subcutaneous injection of 5 µg/kg of AVP (arginine vasopressin), palmitoyl-AVP

and REAL-AVP in accordance with the invention. The data show the mean and SD of measurements from 3 rats.

FIG. 3 shows the cumulative urine output of diabetic rats over 24 hours after subcutaneous injection of 5 μ g/kg of AVP, palmityl-AVP and REAL-AVP in accordance with the invention. The data show the mean and SD of 3 experiments.

FIG. 4 shows the change in blood glucose level in fasted diabetic rats after subcutaneous injection of 0.35 U/kg of insulin compared with subcutaneous injection of 0.35 U/kg of REAL-insulin of the invention. The data show the mean and SD of measurements with 2 rats.

FIG. 5 shows the prolonged effect on blood glucose levels in fasted diabetic rats of subcutaneous injection of 0.5 U/kg insulin compared with subcutaneous injection of 0.5 U/kg REAL-insulin of the invention. The data show the mean and SD of measurements with 2 rats.

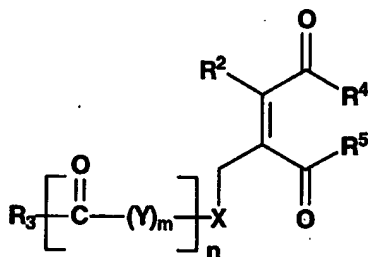
FIG. 6 shows the short-term effect on blood glucose level of fasted diabetic rats after oral administration of 10 U/kg REAL-insulin, insulin and placebo. The data show the mean and SD of measurements with four rats.

Detailed Description of the Invention

In accordance with the present invention, a biologically active amine containing compound (for example an amino acid, peptide or protein) is attached to a lipophilic derivative via a reversible amide bond. The lipophilic group of such a conjugate binds to the apical side of a cell membrane and facilitates the transport of the conjugate through the cell membrane. Once inside the cell membrane, the biologically active amine containing compound is released into the interstitial fluid as the result of hydrolysis of the amide bond.

Pursuant to one aspect of the present invention, there are provided conjugates of the general Formula I

-12-



I

in which R^2 is hydrogen, halo, alkyl, or aryl, wherein the alkyl and aryl groups are optionally substituted with one or more alkoxy, alkoxyalkyl, alkanoyl, nitro, cycloalkyl, alkenyl, alkynyl, alkanoyloxy, alkyl or halogen atoms;

5 R^3 is a lipophilic group;

one of R^4 and R^5 represent a biologically active amino group containing substance selected from the group consisting of amine-containing drugs, natural or unnatural amino acids, peptides and proteins and the other of R^4 and R^5 is OR^6 where R^6 represent hydrogen, an alkali metal or a negative charge;

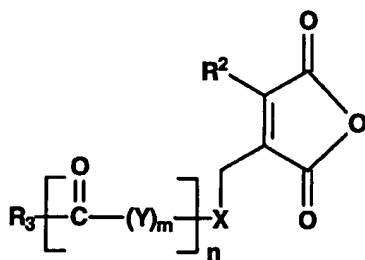
10 X is O or S;

Y is a bridging natural or unnatural amino acid;

n is zero or 1; and

m is an integer from zero to 10.

Pursuant to another aspect of the present invention, there are provided
15 compounds of the general Formula II



II

in which R^2 is hydrogen, halo, alkyl, or aryl, wherein the alkyl and aryl groups are optionally substituted with one or more alkoxy, alkoxyalkyl, alkanoyl, nitro, cycloalkyl, alkenyl, alkynyl, alkanoyloxy, alkyl or halogen atoms;

R^3 is a lipophilic group;

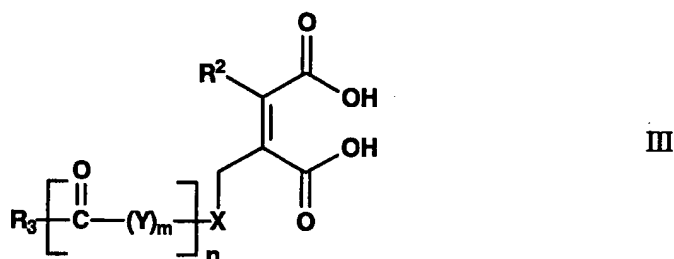
X is O or S;

Y is a bridging natural or unnatural amino acid;

n is zero or 1; and

5 m is an integer from zero to 10.

Pursuant to another aspect of the present invention, there are provided compounds of the general Formula III



or a nontoxic pharmaceutically-acceptable salt thereof,

10 in which R^2 is hydrogen, halo, alkyl, or aryl, wherein the alkyl and aryl groups are optionally substituted with one or more alkoxy, alkoxyalkyl, alkanoyl, nitro, cycloalkyl, alkenyl, alkynyl, alkanoyloxy, alkyl or halogen atoms;

R^3 is a lipophilic group;

X is O or S;

15 Y is a bridging natural or unnatural amino acid;

n is zero or 1; and

m is an integer from zero to 10.

Typical alkyl groups include C_{1-6} alkyl groups including methyl, ethyl, propyl, isopropyl, butyl, isobutyl, *sec*-butyl, *tert*-butyl, pentyl, 2-pentyl, 3-pentyl, neopentyl, hexyl, 2-hexyl, 3-hexyl, 2-methyl-1-pentyl, 3-methyl-1-pentyl, 4-methyl-1-pentyl, and the like.

Typical alkoxy groups include oxygen substituted by any of the alkyl groups mentioned above.

Typical alkoxyalkyl groups include any of the above alkyl groups substituted by an alkoxy group, such as methoxymethyl, ethoxymethyl, propoxymethyl, butoxymethyl, pentoxymethyl, hexoxymethyl, methoxyethyl, methoxypropyl, methoxybutyl, methoxypentyl, methoxyhexyl, and the like.

5 Preferred aryl groups are C₆₋₁₄ aryl groups and typically include phenyl, naphthyl, fluorenyl, phenanthryl, and anthracyl groups.

Typical alkoxy substituted aryl groups include the above aryl groups substituted by one or more of the above alkoxy groups, e.g., 3-methoxyphenyl, 2-ethoxyphenyl, and the like.

10 Typical alkyl substituted aryl groups include any of the above aryl groups substituted by any of the C₁₋₆ alkyl groups, including the group Ph(CH₂)_n, where n is 1-6, for example, tolyl, *o*-, *m*-, and *p*-xylyl, ethylphenyl, 1-propylphenyl, 2-propylphenyl, 1-butylphenyl, 2-butylphenyl, *t*-butylphenyl, 1-pentylphenyl, 2-pentylphenyl, 3-pentylphenyl.

15 Typical alkenyl groups include C₂₋₆ alkenyl groups, e.g. ethenyl, 2-propenyl, isopropenyl, 2-butenyl, 3-butenyl, 4-pentenyl, 3-pentenyl, 2-pentenyl, 5-hexenyl, 4-hexenyl, 3-hexenyl, and 2-hexenyl groups.

20 Typical alkynyl groups include C₂₋₆ alkynyl groups e.g. ethynyl, 2-propenyl, 2-butylnyl, 3-butylnyl, 4-pentylnyl, 3-pentylnyl, 2-pentylnyl, 5-hexynyl, 4-hexynyl, 3-hexynyl, and 2-hexynyl groups.

25 Typical alkenyl or alkynyl substituted aryl groups include any of the above C₆₋₁₄ aryl groups substituted by any of the above C₂₋₆ alkenyl or C₂₋₆ alkynyl groups, e.g., ethenylphenyl, 1-propenylphenyl, 2-propenylphenyl, 1-butenylphenyl, 2-butenylphenyl, 1-pentenylphenyl, 2-pentenylphenyl, 3-pentenylphenyl, 1-hexenylphenyl, 2-hexenylphenyl, 3-hexenylphenyl, ethynylphenyl, 1-propynylphenyl, 2-propynylphenyl, 1-butylnylphenyl, 2-butylnylphenyl, 1-pentylnylphenyl, 2-pentylnylphenyl, 3-pentylnylphenyl, 1-hexynylphenyl, 2-hexynylphenyl, 3-hexynylphenyl groups.

Typical halo groups include fluorine, chlorine, bromine, and iodine.

Typical halo substituted alkyl groups include C₁₋₆ alkyl groups substituted by one or more fluorine, chlorine, bromine, or iodine atoms, e.g., fluoromethyl, difluoromethyl, trifluoromethyl, pentafluoroethyl, 1,1-difluoroethyl, and trichloromethyl groups.

5 Typical alkanoyl groups include C₁₋₅C(O) alkanoyl groups, e.g., acetyl, propionyl, butanoyl, pentanoyl, and hexanoyl groups, or by an arylalkanoyl group, e.g., a C₁₋₅C(O) alkanoyl group substituted by any of the above aryl groups.

10 Typical cycloalkyl groups include C₃₋₈ cycloalkyl groups including cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl groups.

Pursuant to yet another aspect of the present invention, there are provided methods of forming conjugates of general Formula I from compounds of general Formula II and an amino group containing substance.

15 Pursuant to yet another aspect of the present invention, there are provided methods of forming compounds of general Formula II from maleic acid derivatives and thiols and alcohols.

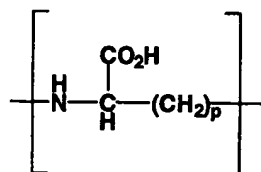
20 Pursuant to yet another aspect of the present invention, methods for increasing the absorption or prolonging blood and tissue retention in a mammal of a biologically active amino group containing substance are provided, in which a conjugate of general Formula I is administered to the mammal (for example, in the form of emulsions, nanoparticles (e.g. solid lipid nanoparticles), liposomes, microspheres, microcapsules, aerosols, through inhalation, and transdermal dosage forms).

25 Pursuant to yet another aspect of the present invention there are provided methods for increasing the delivery of hydrophilic amine containing compounds to the inside of a cell having a mucosal barrier, in which a conjugate of general Formula I is contacted with the cell whereby the conjugate penetrates the mucosal barrier of the cell and the free amine is liberated by hydrolysis of an amide bond.

30 The term "lipophilic group" as used herein refers to either a naturally occurring lipid per se, a hydrophobic branched or unbranched hydrocarbon

comprising about 4 to about 26 carbon atoms, preferably about 5 to about 19 carbon atoms, a fatty acid or ester thereof, or a surfactant. Suitable lipophilic groups include, but are not limited to, long chain alkanoyl groups including: palmityl ($C_{15}H_{31}$), oleyl ($C_{15}H_{29}$), stearyl ($C_{17}H_{35}$), lauryl ($C_{11}H_{23}$), cholesteryl, and myristyl ($C_{13}H_{27}$).

The term "natural or unnatural amino acid" as used herein refers to any of the 21 naturally occurring amino acids as well as D-form amino acids, blocked L- and D-form amino acids such as those blocked by amidation or acylation, substituted amino acids (e.g., those substituted with a sterically hindered alkyl group or a cycloalkyl group such as cyclopropyl or cyclobutyl) in which the substitution introduces a conformational restraint in the amino acid. The preferred naturally occurring amino acids for use in the present invention as amino acids or components of a peptide or protein are alanine, arginine, asparagine, aspartic acid, citrulline, cysteine, cystine, γ -glutamic acid, glutamine, glycine, histidine, isoleucine, norleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, hydroxyproline, serine, threonine, tryptophan, tyrosine, valine, γ -carboxyglutamate, or O-phosphoserine. The preferred non-naturally occurring amino acids for use in the present invention as amino acids or components of peptides or proteins are any of the β -amino acids, e.g., β -alanine, α -amino butyric acid, γ -amino butyric acid, γ -(aminophenyl) butyric acid, α -amino isobutyric acid, ϵ -amino caproic acid, 7-amino heptanoic acid, amino benzoic acid, aminophenyl acetic acid, aminophenyl butyric acid, cysteine (ACM), methionine sulfone, phenylglycine, norvaline, ornithine, δ -ornithine, *p*-nitro-phenylalanine, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid and thioproline. Also contemplated are amino acid derivatives of the Formula:



where p is 1-10.

The term "biologically active amino group containing substance" as used herein refers to any substance having biological activity when introduced inside a cell and including in its structure a primary or secondary amine capable of forming an amide bond through acylation. Substances which do not include a primary or secondary amine may be suitably derivatized so as to be amenable to conjugation with compounds of general Formula II or III. For example, compounds having carboxy groups may be reacted with a suitable diamine, e.g. a C₂-C₁₀ diamine such as ethylene diamine, propylene diamine, 1,4-diaminobutane, spermine or spermidine and the like in the presence of a compound of general Formula II or III and a water-soluble carbodimide (e.g., EDC) coupling reagent. In this way the diamine serves as means for coupling a biologically active compound, which does not include a primary or second amine, to a compound of general Formula II or III via amide bond formation.

Preferred amine-containing drugs include, but are not limited to, tyramine, arginine vasopressin, insulin (Czech, M.P., *Ann. Rev. Biochem.* 46:359 (1977)), calcitonin (Brown, E.M. and Aurbach, G.D., *Vitam. Horm.* 38:236 (1980)), desmopressin (Vavra, *et al.*, *J. Pharmacol. Exp. Ther.* 188:241 (1974)), interferon- α , - β , and γ (Stiem, E.R., *Ann. Rev. Inter. Med.* 96:80-93 (1982)), interleukin-2, -3, -4, -6, and -11 (Kluth, D.C. and Rees, A.J., *Semin. Nephrol.* 16:576-582 (1996)); Holyoake, T.L., *Blood Rev.* 10:169-200 (1996)), G-CSF (Spiekermann, K., *et al.*, *Leukemia* 11:466-478 (1997)), GM-CSF (Jonuleit, H., *et al.*, *Arch. Dermatol. Res.* 289:1-8 (1996)), human growth hormone (Strobl, J.S. and Thomas, M.J., *Pharmacol. Rev.* 46:1-34 (1994)), erythropoietin (Spivak, J.L., *Semin. Hematol.* 30:2-11 (1993)), vasopressin (Schroder, E. and Lubke, K., *The Peptide* 2:336-350 (1966)), octreotide (Sheppard, M.C. and Stewart, P.M., *Metabolism: Clinical and Experimental* 45:63-64 (1996)), aprotinin (Haderland, G. and McConn, R., *Fed. Proc.* 38:2760-2767 (1979)), oxytocin (Nachtmann, F., *et al.*, in *Anal. Prof. Drug Subst.*, Vol. 10, Florey, K., ed., Academic Press, New

York, NY (1981), pp. 563-600), β -TGF (Moses, H.L. and Serra, R., *Curr. Opin. Genet. Dev.* 6:581-586 (1996)), BDNF (Apfel, S.C. and Kessler, J.A., *Baillieres. Clin. Neurol.* 4:593-606 (1995)), b-FGF (Bikfalvi, A., *et al.*, *Endocr. Rev.* 18:26-45 (1997)), PDGF (Hughes, A.D., *et al.*, *Gen. Pharmacol.* 27:1079-1089 (1996)),
5 TNF (Majno, P.E., *et al.*, *Swiss. Surg.* 4:182-185 (1995)), atrial natriuretic peptide (Nakao, K., *Curr. Opin. Nephrol. Hypertens.* 2:45-50 (1993)), relaxin (Schwabe, C., *et al.*, *Recent Progr. Horm. Res.* 34:123-211 (1978)), amylin (Rink, T.J., *et al.*, *Trends. Pharmacol. Sci.* 14:113-118 (1993)), deoxyribonuclease (Laskowski, in *The Enzymes*, Vol. 2, Boyer, P.D., ed., Academic Press, New York, NY
10 (1971), pp. 289-311), EGF (Carpenter, G., *Curr. Opin. Cell. Biol.* 5:261-264 (1993)), hirudin (Markwardt, *Methods. Enzymol.* 19:924 (1970)), neocarcinostatin (Dedon, P.C. and Goldberg, I.H., *Chem. Res. Toxicol.* 311-332 (1992)), hemoregulatory peptide (Paukovits, W.R., *et al.*, *Cancer Treat. Rev.* 17:347-354 (1990)), and somatostatin (Moss, R.L., *Ann. Rev. Physiol.* 41:617
15 (1979)).

For purposes of the present invention, the term "peptide" refers to natural or unnatural amino acid chains comprising two to 100 amino acids and the term "protein" to natural or unnatural amino acid chains comprising more than 100 amino acids. The proteins and peptides may be isolated from natural sources or
20 prepared by means well known in the art, such as recombinant DNA technology or solid-state synthesis. It is contemplated that the peptides and proteins used in accordance with the present invention may comprise only naturally-occurring L-amino acids, combinations of L-amino acids and other amino acids (including D-amino acids and modified amino acids), or only amino acids other than
25 L-amino acids. In order to form a conjugate of general Formula I, the peptide or protein must bear at least one reactive amine group. The reactive amine group may be part of an amino acid side chain, or a terminal amino group of the peptide or protein backbone, or introduced by chemical modification of functional groups in peptide or protein molecules. Peptides can be homo- or hetero-peptides and

can include natural amino acids, synthetic amino acids, or any combination thereof.

Also included within the scope of the present invention are nontoxic pharmaceutically-acceptable salts of the compounds of the invention. In particular, the alkali metal carboxylates, formed by known methods such as the addition of an alkali metal halide to the corresponding carboxylic acid, are contemplated. Such salts include the sodium, potassium, lithium and ammonium salts.

The term "negative charge" as used herein refers to any unsolvated, solvated or complexed lone pair of electrons capable of providing anionic character to a carboxylate group.

The term "alkali metal" as used herein refers to any of the Group I or Group II metals, for example sodium, potassium, lithium, calcium, and magnesium.

The preferred animal subject of the present invention is a mammal. The term "mammal" refers to an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human patients.

The term "treating" refers to the administration to subjects of a lipidization conjugate for purposes which can include prevention, amelioration, or cure of a disease or condition.

Medicaments are considered to be provided "in combination" with one another if they are provided to the patient concurrently or if the time between the administration of each medicament is such as to permit an overlap of biological activity.

In one preferred embodiment, at least one conjugate is present or administered as part of a pharmaceutical composition.

Pharmaceutical compositions for administration according to the present invention can comprise at least one conjugate according to the present invention in a pharmaceutically acceptable form optionally combined with a pharmaceutically acceptable carrier. These compositions can be administered by

any means that achieve their intended purposes. For example, administration may be by oral, parenteral, subcutaneous, intravenous, intramuscular, intra-peritoneal, transdermal, intrathecal, intracranial or intranasal routes. The dosage administered will be dependent on the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. Amounts and regimens for administration according to the present invention can be determined readily by those with ordinary skill in the art of clinical treatment.

The form of administration may also include emulsions, nanoparticles (e.g., solid lipid nanoparticles), liposomes, microspheres, microcapsules, aerosols, through inhalation, and transdermal dosage forms.

Suitable formulations for parenteral administration include aqueous solutions of the compounds in water-soluble form. In addition, suspensions of the compounds as appropriately oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, aqueous solutions and/or suspensions may also contain stabilizers and/or buffers, such as borate buffer and the like.

Pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. Thus pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

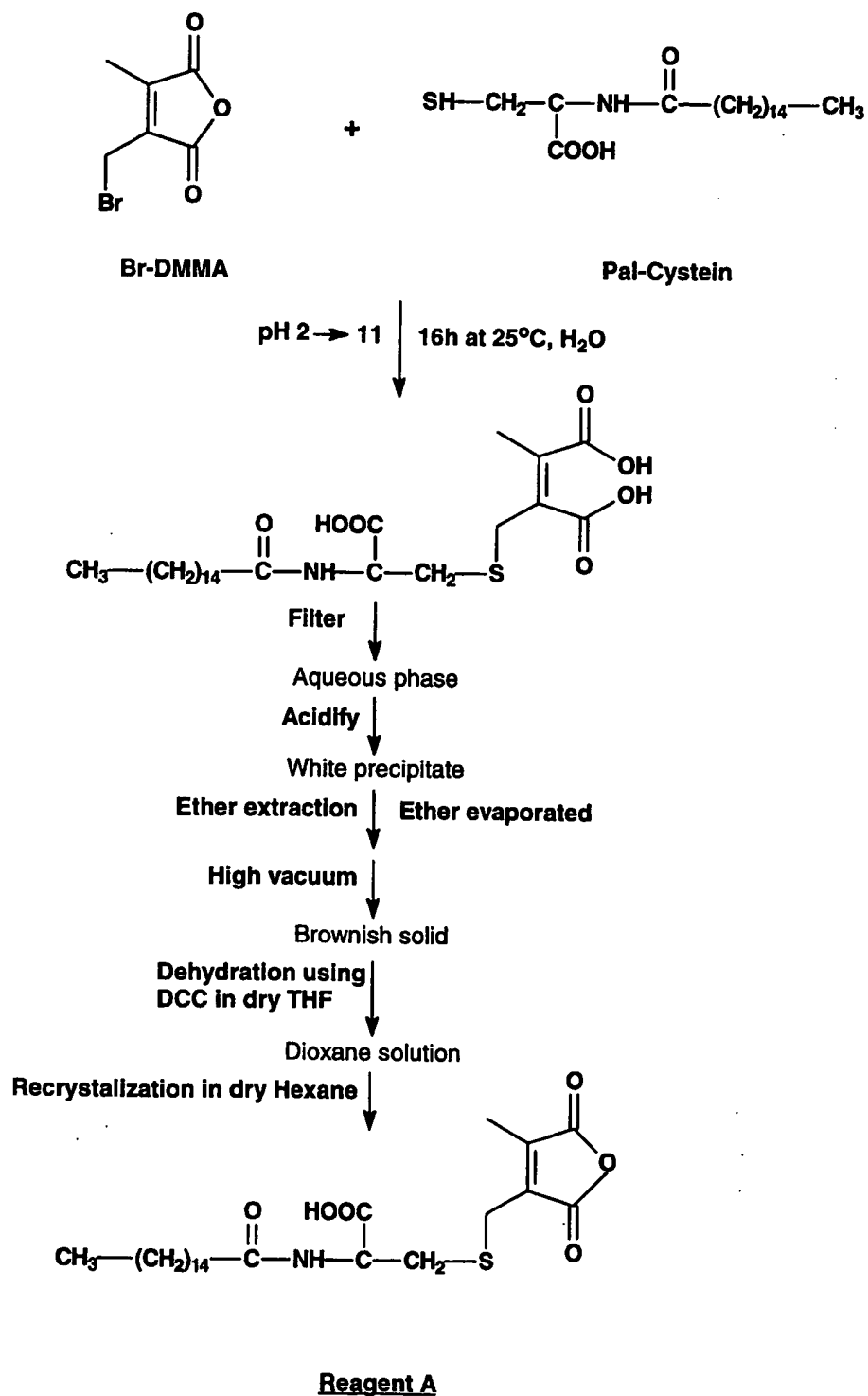
Suitable excipients are, e.g., fillers such as saccharide, lactose, sucrose, mannitol or sorbitol; cellulose preparations and/or calcium phosphates, such as tricalcium phosphate or calcium hydrogen phosphate; as well as binders such as

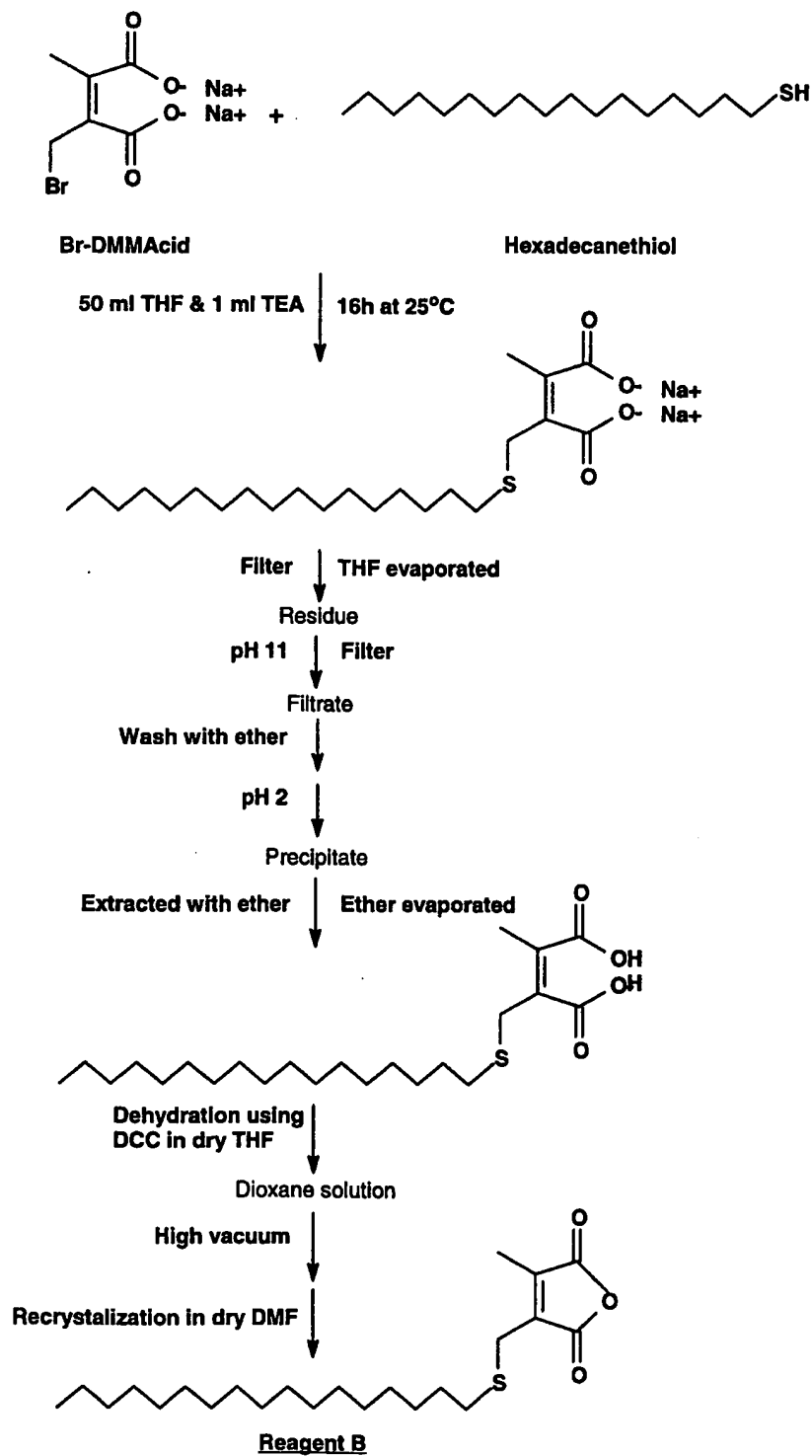
starch paste, using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, tagaranth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents can be added such as the above-mentioned starches and also carboxymethyl starch, cross-linked polyvinyl pyrrolidone, agar or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate, and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose concentrated saccharide solutions can be used which can optionally contain gum arabic, talc, polyvinyl pyrrolidone, polyethylene glycol, and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetyl cellulose phthalate or hydroxypropylmethyl cellulose phthalate are used. Coatings may also be provided to protect the lipidization conjugates of the present invention from premature exposure to an acidic environment sufficient to hydrolyze the amide bond formed between the active drug, peptide or protein and the carrier. See U.S. Patent Nos. 4,786,505 and 4,853,230 for methods of preparing dosage units with cores that are protected from gastric acid. Preferably, the core is neutral or basic. Basic cores contain one or more alkaline reacting compounds such as those described in U.S. Patent Nos. 4,786,505 and 4,853,230. Dystuffs or pigments can be added to the tablets or dragee coatings, for example, for identification in order to characterize combinations of active compound doses.

Other pharmaceutical preparations which can be used include, but are not limited to, oral push-fit capsules made of gelatin, rectal suppositories, inhalation formulations for oral and/or nasal administration, nasal or rectal creams or ointments optionally combined with a pharmaceutically acceptable carrier, penetration enhancer, excipient, and/or filler. Penetration enhancers suitable for

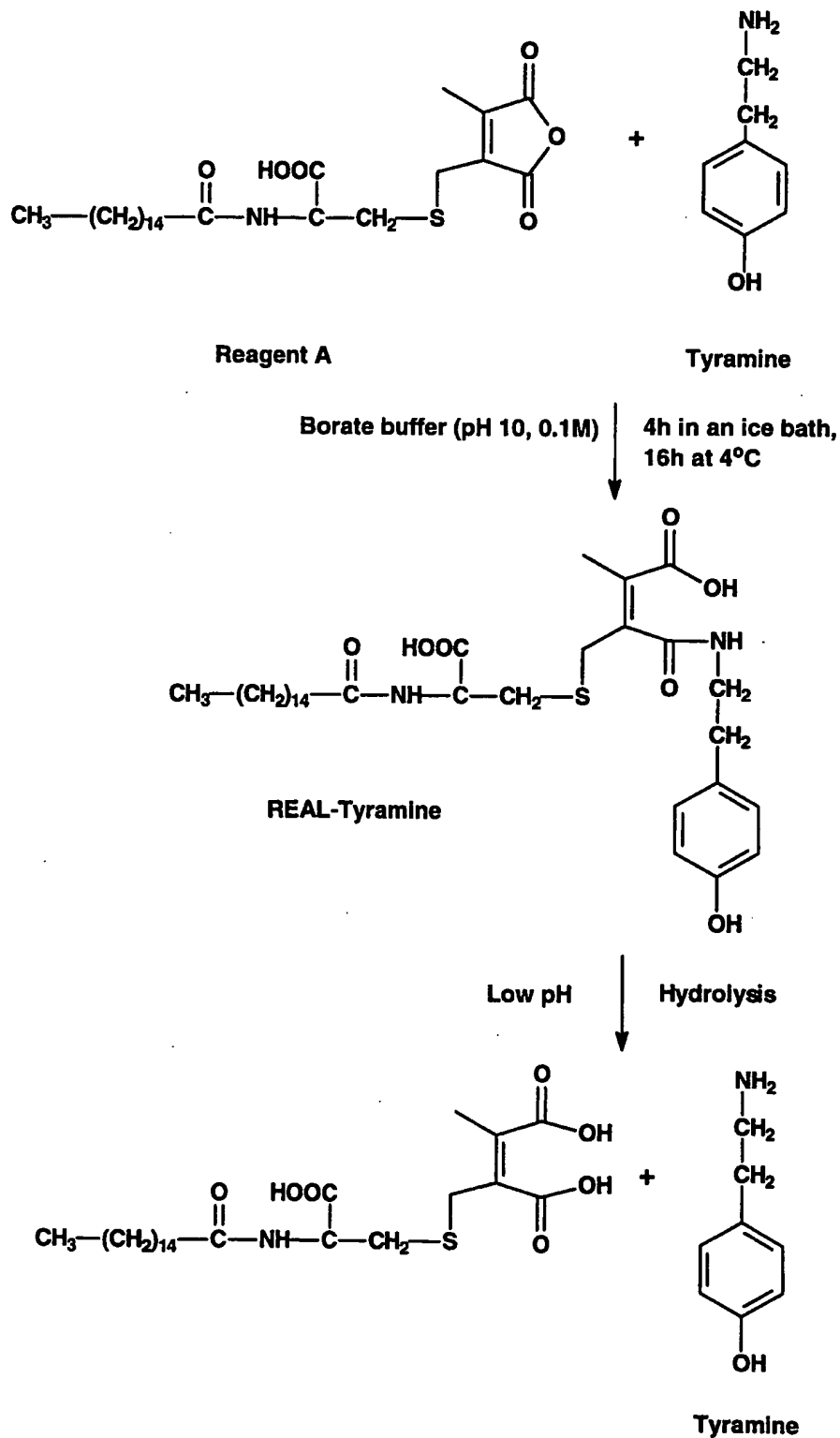
use include cationic, anionic, amphoteric and neutral penetration enhancers such as benzalkonium chloride, chlorbutanol, AZONE and others known in the art.

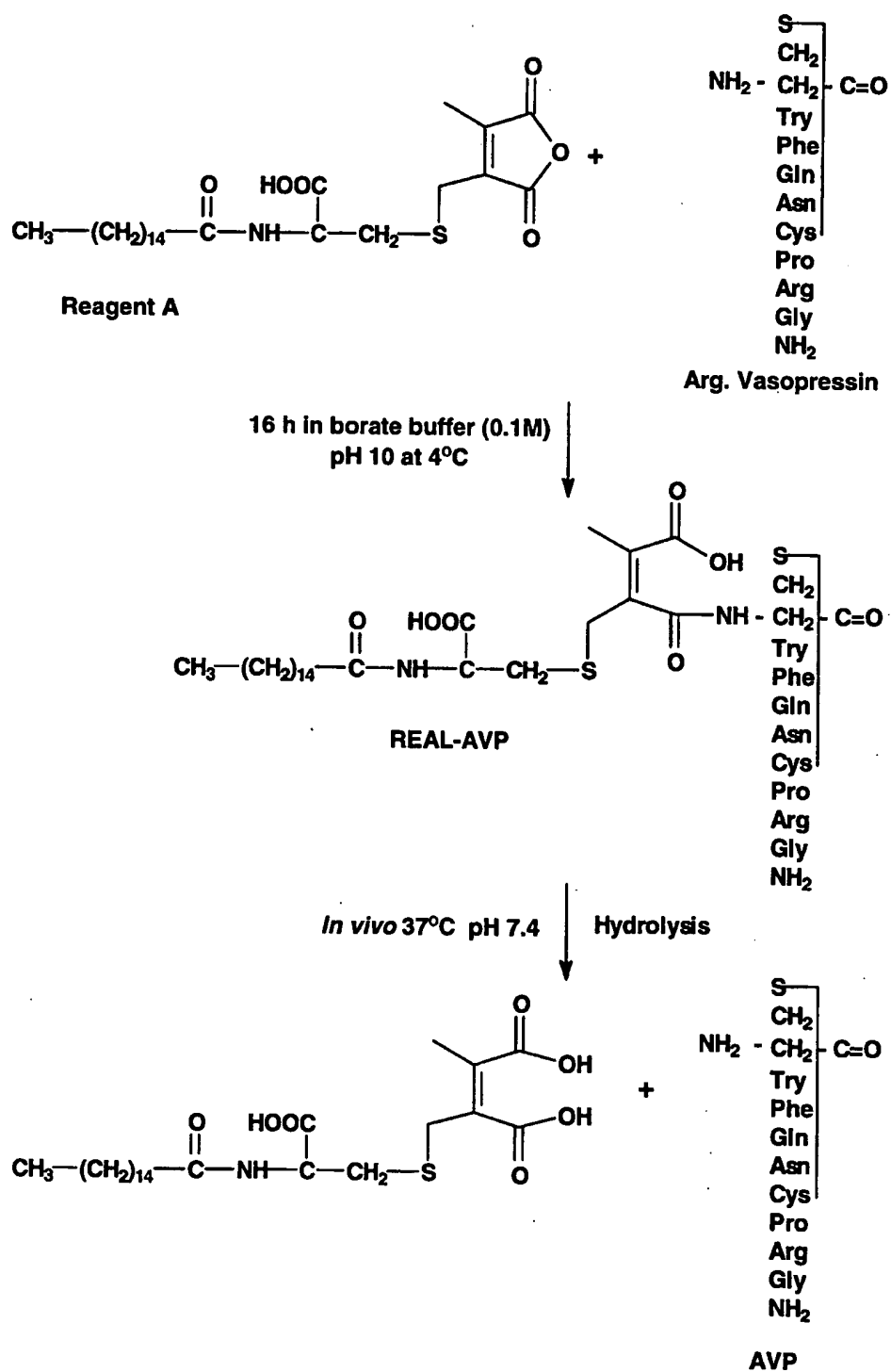
The synthesis of exemplary compounds of general Formula II is illustrated in Schemes 1 and 2. In general, a bromomethyl maleic anhydride derivative, or its maleate salt, is allowed to react with an alcohol or thiol-bearing lipophilic group to form an ether or a thiol ether of general Formula III. The alcohol or thiol-bearing lipophilic group optionally includes a bridging natural or unnatural amino acid moiety bridging the oxygen or sulfur atom and the carbonyl bound to lipophilic group. The bridging natural or unnatural amino acid moiety may be connected to either the oxygen or sulfur atom or the carbonyl bound to lipophilic group at the amino terminus, carboxyl terminus or side chain of the amino acid. With reference to Scheme I, Pal-cystein effectively includes a glycine bridge bound to the carbonyl at the amino terminus and to the sulfur atom via the side chain. The use of hexadecanethiol as in Scheme 2 represents the formation of compounds of Formula III without the bridging natural or unnatural amino acid. The product of general Formula III is then subjected to dehydrating conditions to reform the maleic anhydride now substituted via the ether or thioether linkage with a lipophilic group, giving compounds of general Formula II. Those skilled in the art will appreciate a variety of alternative synthetic schemes capable of arriving at the desired compounds.

Scheme 1 - Synthesis of the reagent A

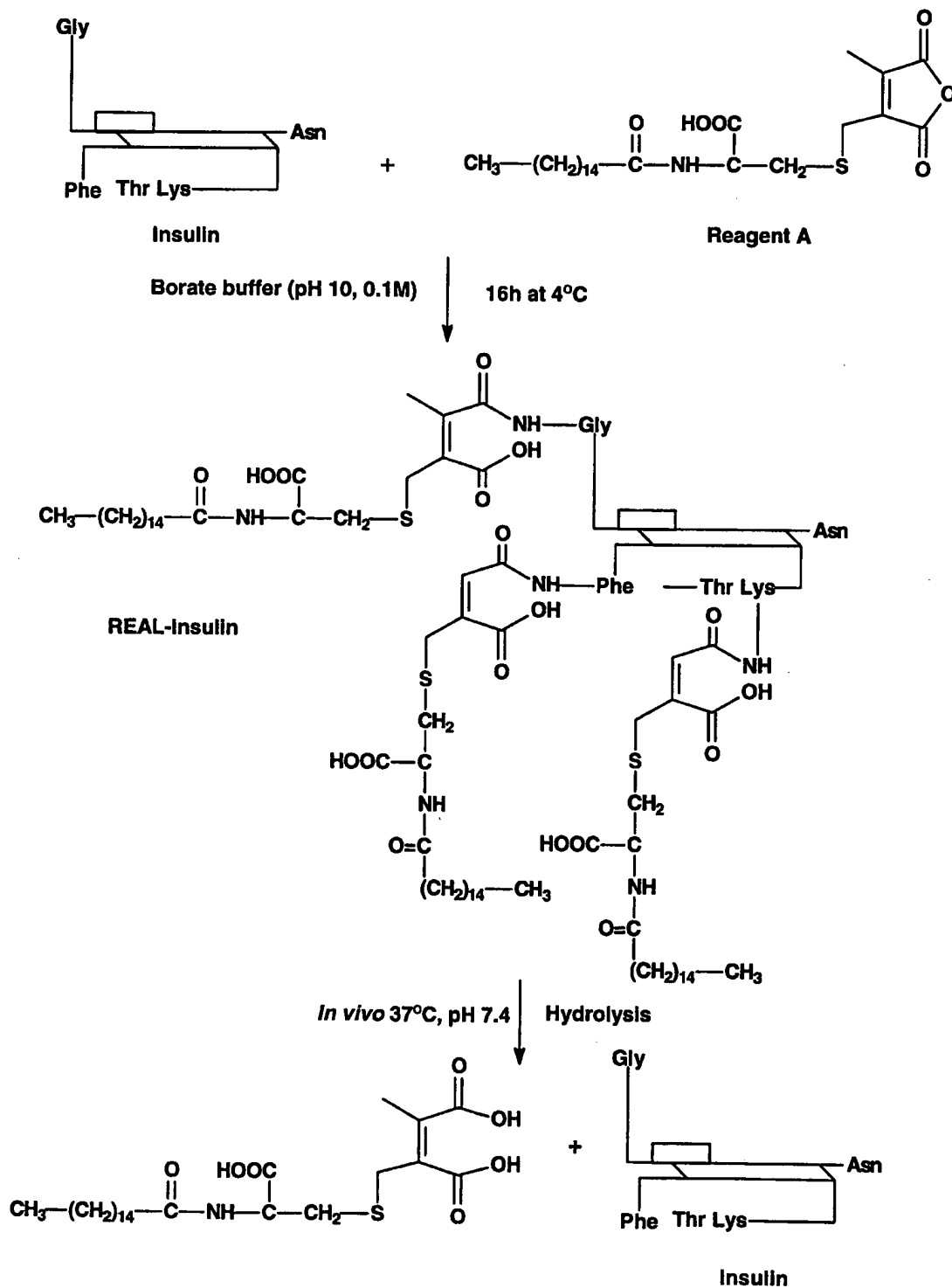
Scheme 2 - Synthesis of the reagent B

Schemes 3-5 outline the synthesis of exemplary pH sensitive lipidization conjugates in accordance with the present invention. In general, an amine containing drug, amino acid, peptide or protein is allowed to react with a compound of Formula II to form an amide of Formula I. The amide bond is formed under alkaline conditions, preferably in a buffered aqueous solution. At lower pH, including pH typically found *in vivo*, the amide bond is hydrolyzed releasing the free amine and a compound of Formula III. Reversible amide bond formation provides a mechanism for the conjugation of a hydrophilic amine with a lipidization reagent at one pH and the release of that amine from the lipidization reagent at a lower pH.

Scheme 3 -Lipidization of Tyramine (REAL-Tyramine)

Scheme 4 - Lipidization of Arg. Vasopressin (REAL-AVP)

Scheme 5 - Lipidization of Insulin (REAL-Insulin)



Examples

Example 1. Synthesis of 3-S-(N-Palmityl cysteinyl) methyl, 2-methyl maleic anhydride, Reagent A (Scheme 1)

5 The pyridine disulfide derivative of N-palmityl-cysteine (Pal-CPD) was obtained by known methods. Pal-CPD was synthesized according to the procedure of Ekrami *et al.*, *FEBS Letters* 371:283-286 (1995). Pal -CPD (0.7g, 0.0015mol) was dissolved in 10ml NaOH pH 11. Dithiothreitol (DTT) (0.9g, 0.006mol) was dissolved in 5ml water. The Pal-CPD solution was added drop wise to the DTT solution under continuous stirring at room temperature. After 10 2h, the reaction was terminated. The pH of the mixture was adjusted to 3 using 0.01N HCl wherein a white precipitate occurred (Pal-cysteine). The precipitate was washed 5 times using diluted HCl to remove the excess amount of DTT.

The starting material 3-bromomethyl, 2-methyl maleic anhydride (Br-DMMA) was obtained by the addition of one equivalent of bromide radical to 15 2,3-dimethyl maleic anhydride (DMMA). Accordingly, DMMA (1.5g, 0.012mol), NBS (2.3g, 0.013mol), benzoyl peroxide (0.3g, 0.0012mol) and magnesium oxide (0.02g, 0.0005mol) were heated in 40ml chloroform under reflux for 4h. The mixture was filtered and chloroform was evaporated under reduced pressure. To the brown residue 40 ml carbon tetrachloride was added 20 and filtered. The filtrate was collected and the solvent removed under reduced pressure. A clear oil with a light greenish color was obtained which was solidified after storage at 4°C.

With reference to Scheme 1, Br-DMMA is reacted with Pal-cysteine to afford a Pal-cysteine thiol ether of Formula III where R¹ is hydrogen, R² is 25 methyl, R³ is palmityl (C₁₅H₃₁), X is sulfur, Y is a glycine radical (-NHCH(CO₂H)-), n=1 and m=1. The reaction is carried out by adding Br-DMMA (0.3g, 0.0014mol) directly to a suspension of Pal-cysteine in 30ml diluted HCl at room temperature. The pH of the mixture was gradually adjusted

to 7, 9 and finally 11, using 1N NaOH. The pH of the mixture was stabilized after 2h at pH 11. After 16h stirring at 25°C, the mixture was filtered and the filtrate acidified using 1N HCl. A white precipitate occurred which was extracted with ether. Ether was evaporated under reduced pressure. The remaining greenish oil was dried under high vacuum. 3-S-(N-Palmityl cysteinyl) methyl, 2-methyl maleic acid (420 mg, 0.84mmol) was obtained with a melting point of 60-63°C. Molar yield was 56%.

3-S-(N-Palmityl cysteinyl) methyl, 2-methyl maleic acid (420 mg, 0.84mmol) was dissolved in 5ml dry THF. N,N-Dicyclohexylcarbodiimide (DCC) (692mg, 3.36mmol) was dissolved in 1ml dry THF and added to the above solution in an ice bath. The reaction was stirred in an ice bath for 5h and then filtered. The filtrate was collected and THF removed under reduced pressure. The residue (brownish solid) was dissolved in 1.5ml dry dioxane and filtered. The filtrate was added to 30 ml cold dry hexane and kept at 4°C for 16h. The precipitate obtained was washed using cold dry hexane and applied to high vacuum in order to remove the solvent. A light brownish product (reagent A) was obtained with a melting point of 46-49°C. Molar yield was 54%.

Example 2. Synthesis of 3S-(hexadecanyl) methyl 2-methyl maleic anhydride, Reagent B (scheme 2)

With reference to Scheme 2, Br-DMMA is reacted with hexadecanethiol to afford a thiol ether of Formula III where R² is methyl, R³ is hexadecane (C₁₆H₃₃), n=0 and m=0. Under dehydrating conditions, the anhydride reagent B of Formula II is obtained where R², R³, n and m are as above.

Accordingly, as outlined in Scheme 2, Br-DMMA (0.5g, 0.0025mol) was hydrolyzed in 10ml water at pH 8 and added to 0.63g hexadecanethiol (0.0025mol) dissolved in 50ml THF. Triethylamine (1ml) was added to the mixture and stirred for 16h at room temperature. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure. The residue obtained was dissolved in diluted NaOH solution (pH 11) and washed with ether

(3 x 20mL). The filtrate was adjusted to pH 2 using 1 N HCl and a white precipitate occurred. The precipitate was extracted into ether and the ether removed under reduced pressure. The final product, 3-(hexadecanylthio)methyl 2-methyl maleic acid, was dried under high vacuum.

5 3-(Hexadecanylthio)methyl 2-methyl maleic acid was dehydrated to give reagent B using the same procedure as previously described for reagent A. Reagent B was dissolved in hot DMF and kept at 4°C for 16 h. A white precipitate was occurred which was washed using cold DMF. The solvent was removed under high vacuum. A white powder (73mg) was obtained with a molar
10 yield of 16%.

Example 3. Preparation of Reversibly Lipidized Tyramine (REAL-Tyramine) Using Reagent A (Scheme 3)

Reagent A (2mg, 0.00426mmol) was dissolved in 60μl dry DMF and added to 0.2mg (0.00146mmol) tyramine in 200μl borate buffer USP (pH 10, 0.1M) in an ice bath. The reaction was carried out for 4h in an ice bath and 16h
15 at 4°C.

Example 4. Determination of pH Sensitivity of REAL-Tyramine

The pH dependence of amide bond formation was determined by monitoring the concentration of free tyramine. Phosphate buffers 1M at pH 6, 7
20 & 8 were prepared. REAL-tyramine was diluted 1:2 using these buffers. The stock solution of tyramine was also diluted to have the same concentration of REAL-tyramine and used as control. Samples were incubated at 37°C. Fluorescence of the free tyramine released from REAL-tyramine was determined at different time points using fluorescamine reaction.

25 After lipidization of tyramine, the concentration of free tyramine decreases up to 15% of the original concentration. Incubation of the REAL-tyramine at low pH resulted in an increase in the concentration of free tyramine

indicating the cleavage of the amide bond. The rate of hydrolysis of the amide bond was dependent on the pH, (pH 6 > pH 7 > pH 8). After 1 h incubation of REAL-tyramine at pH 6 amide bond was almost fully hydrolyzed however, at pH 7 about 45% and at pH 8 only 7% of the amide bond of REAL-tyramine was hydrolyzed (Fig. 1).

Example 5. Preparation of Reversibly Lipidized AVP (REAL-AVP) Using Reagent A (Scheme 4)

Arginine vasopressin (AVP) (0.5mg) was dissolved in 1ml borate buffer (pH 10, 0.1M). An aliquot of 0.5ml (0.25mg, 0.207 μ mol) of this solution was reacted in an ice bath with 1 mg (2.1 μ mol) reagent A dissolved in 50 μ l dry dimethylformamide (DMF). The mixture was stirred for 16h at 4°C. The final concentration of REAL-AVP was 0.455mg/ml.

Example 6. In Vivo Effect of REAL-AVP in Vasopressin Deficient Brattleboro Rats

REAL-AVP was injected subcutaneously in animals (5 μ g / kg) and urine was collected at different time points. Figure 2 shows the cumulative volume of urine during the first 8h after injection. AVP and Pal-AVP have similar effects with a delay in urine excretion of 4h. A longer delay in urine excretion, up to 6h, was observed after injection of REAL-AVP. Direct lipidization of AVP to palmitic acid, Pal-AVP, was not as effective as REAL-AVP. The amount of urine excretion was back to original 24 h after injection of Pal-AVP and AVP. However, the effect of REAL-AVP lasted for 3 days (Fig. 3).

It can be concluded that pH sensitive lipidization of AVP prolongs the biological activity of AVP.

Example 7. Preparation of Reversibly Lipidized Insulin (REAL-Insulin) Using Reagent A (Scheme 5)

Insulin (2mg) was dissolved in 2ml borate buffer (pH 10, 0.1M).
Reagent A (1mg, 2.1 μ mol) was dissolved in 100 μ l DMF and reacted with 1ml
5 (1mg, about 0.14 μ mol) insulin solution in an ice bath. The reaction mixture was
stirred for 24h at 4°C and then dialyzed against 500ml borate buffer (pH 10,
0.01M) for 24h at 4°C. The volume of dialyzed REAL-insulin was adjusted to
2ml using borate buffer (pH 10, 0.1M) to give a concentration of 0.5mg / ml of
REAL-Insulin. The volume of insulin stock solution (1ml) was also adjusted to
10 2ml to give a concentration of 0.5mg / ml.

Example 8. The Effect of REAL-Insulin in Hyperglycemic Rats

Diabetes was induced in Sprague Dawley rats using i.v. injection of
60mg /kg, streptozotocin. Solutions of 0.5 Unit / ml insulin or REAL-insulin in
borate buffer (pH 10, 0.1M) were prepared. Rats were fasted 16h prior to the
15 experiment and were injected subcutaneously 0.5 Unit / kg of insulin or REAL-
insulin. The blood glucose level of rats was monitored at different time points
for 9h. After this time rats were fed and the blood glucose level was measured
after 15h of feeding. The rats were again fasted and blood glucose level was
measured after 16h. The period of fasting and feeding was continued for 3 days.

20 The blood glucose level of rats was increased one week after inducing
diabetes from an average of 100 mg/dl to 420 mg/dl (non-fasted rats). In insulin-
treated rats, significant drop in blood glucose level was observed within the first
hour. However, in REAL-insulin treated rats, there was no changes in blood
glucose level within the first hour and a significant drop in blood glucose was
25 observed first 2h after the injection (Fig. 4). This is possibly due to the time
required for REAL-insulin to be hydrolyzed and to release free insulin. After
injection of insulin the fast blood glucose level of rats was back to original within
24h. However, in the case of the rats treated with REAL-insulin, the drug effect

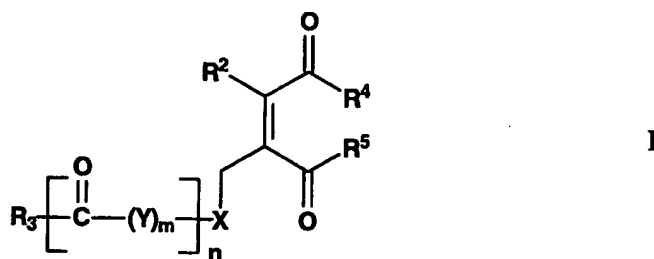
on the fast blood glucose level lasted for 3 days (Fig. 5). Fasted diabetic rats were also administered by oral means 10 U/kg of insulin, REAL-insulin and placebo. Rats were fasted 16h prior to oral administration. A water/oil microemulsion was used as the drug carrier. Fig. 6 shows no significant reduction in blood glucose level was observed after oral administration of insulin or placebo. However, in rats treated with REAL-insulin, a 28% reduction of the blood glucose level was observed in 9 hours.

It can be concluded that by using REAL-insulin, the biological activity of insulin can be prolonged. Using an appropriate formulation, REAL-insulin may be administered orally to reduce blood glucose levels.

Having now fully described this invention, it will be understood to those of ordinary skill in the art that the same can be performed with a wide and equivalent range of conditions, formulations, and other parameters without affecting the scope of the invention or any embodiment thereof. All patents and publications cited herein are fully incorporated by reference herein in their entirety.

What Is Claimed Is:

1. A compound of general Formula I



in which R² is selected from the group consisting of hydrogen, lower alkyl, or aryl, wherein the lower alkyl or aryl are optionally substituted with one or more alkoxy, alkanoyl, nitro, cycloalkyl, alkenyl, alkynyl, acyloxy, lower alkyl or halogen atoms;

R³ is a lipophilic group;

one of R⁴ and R⁵ is a biologically active amino group containing substance selected from the group consisting of an amine-containing drug, a natural or unnatural amino acid, a peptide and a protein and the other of R⁴ and R⁵ is OR⁶ where R⁶ is selected from the group consisting of hydrogen, an alkali metal and a negative charge;

X is oxygen or sulfur;

Y is a bridging natural or unnatural amino acid;

n is zero or 1; and

m is an integer from zero to 10.

2. A compound according to claim 1, wherein R² is methyl and X is sulfur.

3. A compound according to claim 1, wherein n=0, m=0 and R³ is a straight or branched-chain hydrocarbon having 4 to 26 carbon atoms.

4. A compound according to claim 3, wherein said straight or branched-chain hydrocarbon has 5 to 19 carbon atoms.

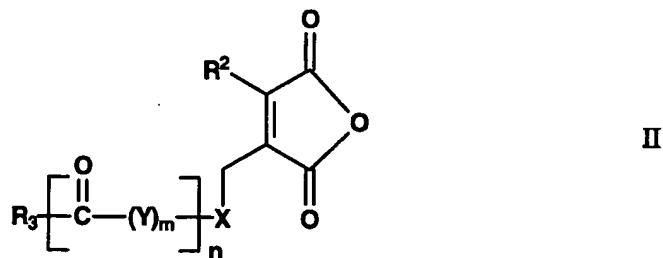
5. A compound according to claim 4, wherein said straight or branched-chain hydrocarbon together with the carbonyl group is selected from the group consisting of palmityl, oleyl, stearyl, lauryl, myristyl, cholate and deoxycholate.

6. A compound according to claim 1, wherein said natural or unnatural amino acid is a naturally-occurring amino acid.

7. A compound according to claim 1, wherein said amine-containing drug is tyramine.

8. A compound according to claim 1, wherein said peptide is selected from the group consisting of Arg. Vasopressin and insulin.

9. A compound of general Formula II



15 in which R^2 is selected from the group consisting of hydrogen, lower alkyl, or aryl, wherein the lower alkyl or aryl are optionally substituted with one or more alkoxy, alkanoyl, nitro, cycloalkyl, alkenyl, alkynyl, acyloxy, lower alkyl or halogen atoms;

R^3 is a lipophilic group;

X is oxygen or sulfur;

Y is a bridging natural or unnatural amino acid;

n is zero or 1; and

m is an integer from zero to 10.

5 10. A compound according to claim 9, wherein R^2 is methyl and X is sulfur.

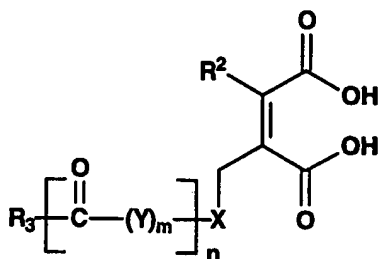
11. A compound according to claim 9, wherein $n=0$, $m=0$ and R^3 is a straight or branched-chain hydrocarbon of 4 to 26 carbon atoms.

10 12. A compound according to claim 11, wherein said straight or branched-chain hydrocarbon is of 5 to 19 carbon atoms.

13. A compound according to claim 11, wherein said straight or branched-chain hydrocarbon together with the neighboring carbonyl group is selected from the group consisting of palmityl, oleyl, stearyl, cholate and deoxycholate.

15 14. A compound according to claim 9, wherein said natural or unnatural amino acid is a naturally-occurring amino acid.

15. A compound of general Formula III



III

or a pharmaceutically acceptable salt thereof;

in which R^2 is selected from the group consisting of hydrogen, lower alkyl, or aryl, wherein the lower alkyl or aryl are optionally substituted with one or more alkoxy, alkanoyl, nitro, cycloalkyl, alkenyl, alkynyl, acyloxy, lower alkyl or halogen atoms;

R^3 is a lipophilic group;

X is oxygen or sulfur;

Y is a bridging natural or unnatural amino acid;

n is zero or 1; and

m is an integer from zero to 10.

16. A compound according to claim 15, wherein R^2 is methyl and X is sulfur.

17. A compound according to claim 15, wherein $n=0$, $m=0$ and R^3 is a straight or branched-chain hydrocarbon of 4 to 26 carbon atoms.

18. A compound according to claim 17, wherein said straight or branched-chain hydrocarbon is of 5 to 19 carbon atoms.

19. A compound according to claim 18, wherein said straight or branched-chain hydrocarbon together with the neighboring carbonyl group is selected from the group consisting of lauryl, myristyl, palmityl, oleyl, stearyl, cholate and deoxycholate.

20. A compound according to claim 15, wherein said natural or unnatural amino acid is a naturally-occurring amino acid.

21. A method of increasing the cellular absorption of a amine-containing substance selected from the group consisting of an amine-containing

drug, a peptide and a protein comprising administering a compound of claim 1 to said cells.

5 22. A method of prolonging blood and tissue retention in a mammal of a biologically active amine-containing compound selected from the group consisting of amine-containing drugs, peptides and proteins comprising administering to said mammal a compound of claim 1.

10 23. A method of forming a compound of claim 1, comprising reacting a biologically active amino group-containing substance selected from the group consisting of an amine-containing drug, a peptide, and a protein with a compound of claim 9, under conditions whereby the compound of claim 1 is obtained.

15 24. A method of delivering a biologically active amino group-containing substance to the interior of a cell, comprising
 exposing said cell to the compound of claim 1, whereby said compound is absorbed by said cell and is exposed to a pH within said cell low enough to hydrolyze an amide bond and release said biologically active amino group-containing substance.

 25. A pharmaceutical composition comprising:
 (a) an effective amount of a compound of claim 1; and
 (b) a pharmaceutically acceptable carrier.

20 26. A pharmaceutical preparation according to claim 25, wherein said composition comprises an enteric coating which protects said compound from amide bond hydrolysis, thereby preventing release of said biologically active amino group containing substance until said coating is removed or dissolved.

1/6

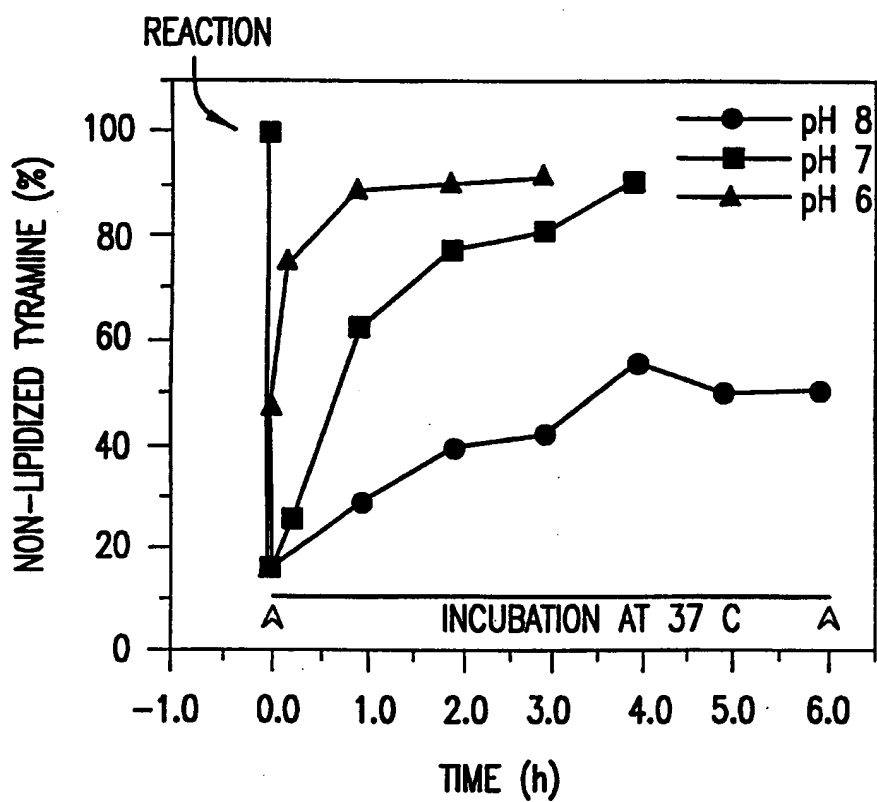


FIG.1

2/6

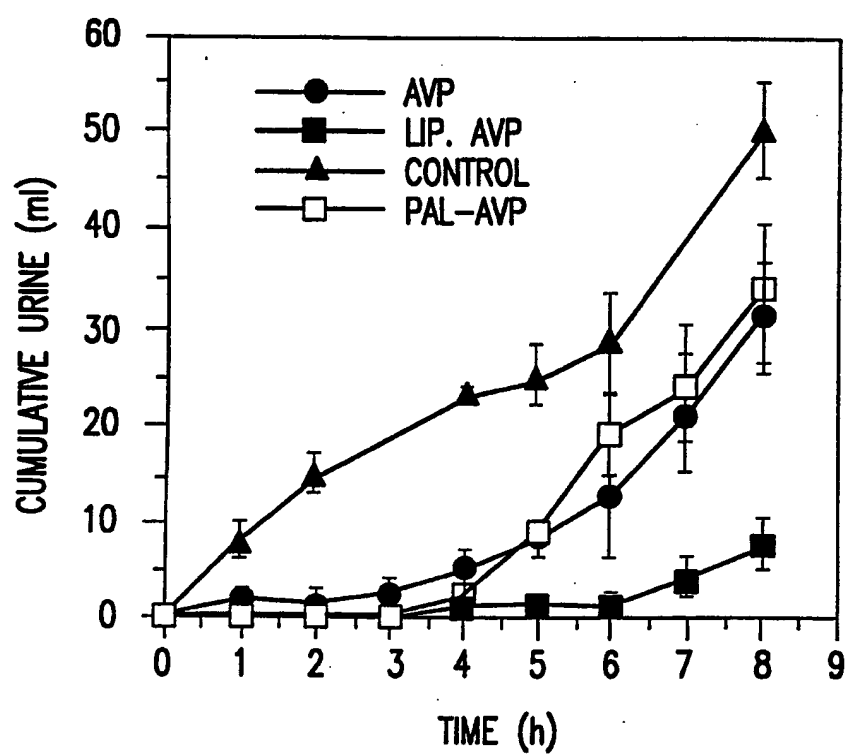


FIG.2

3/6

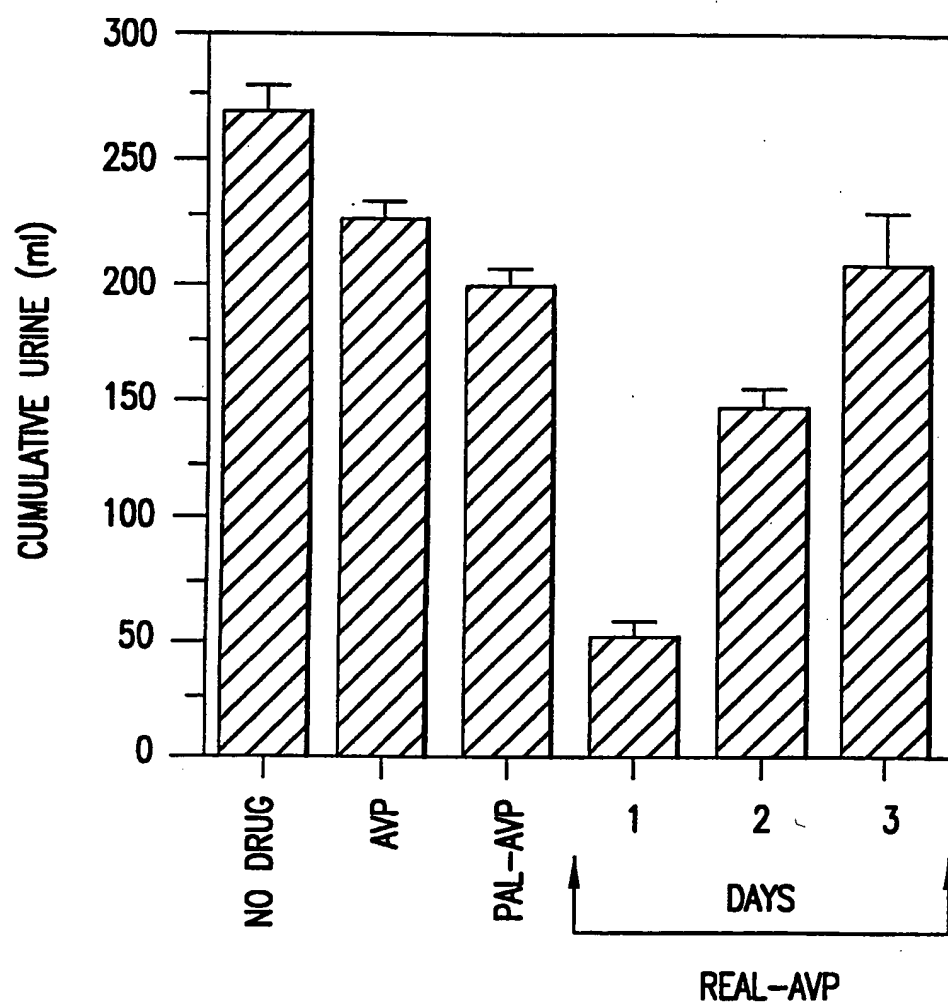


FIG.3

4/6

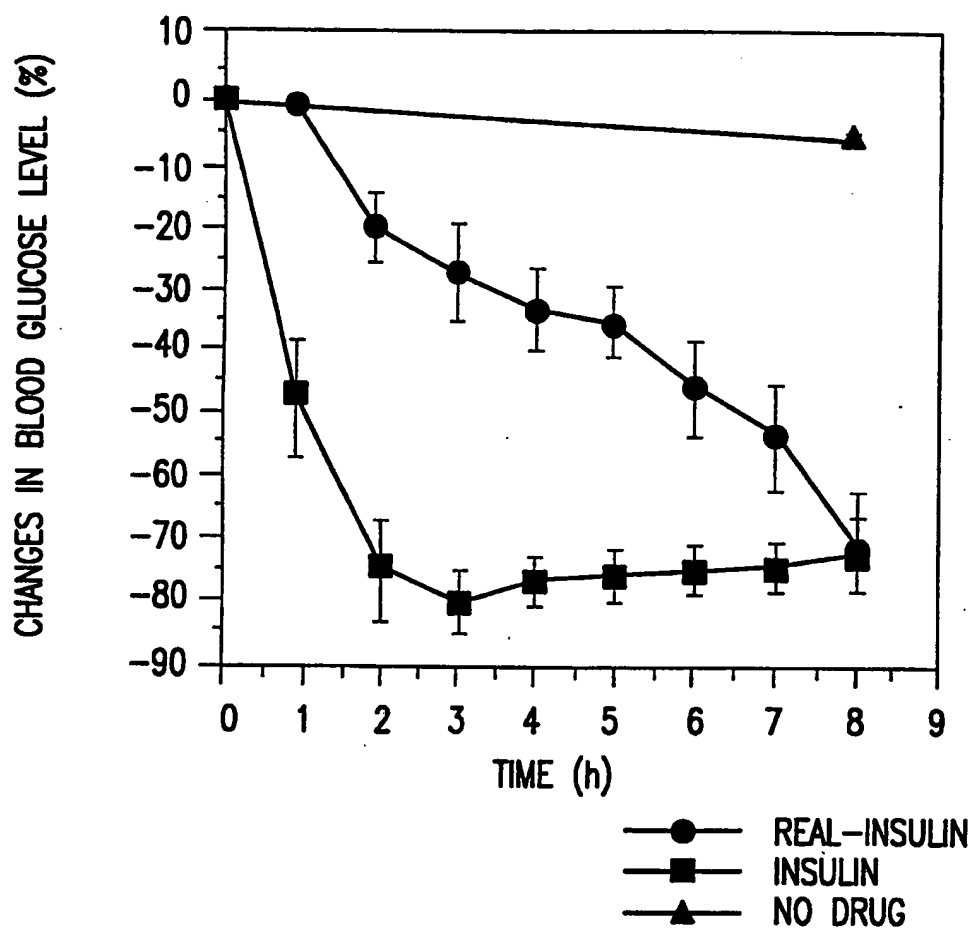


FIG.4

5/6

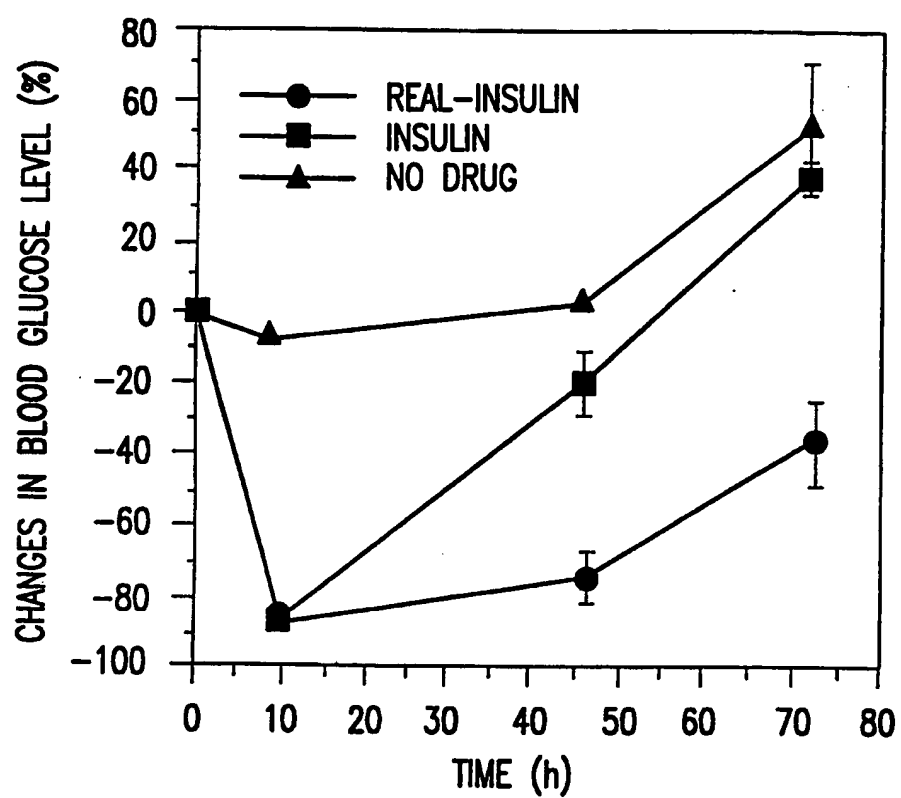


FIG.5

6/6

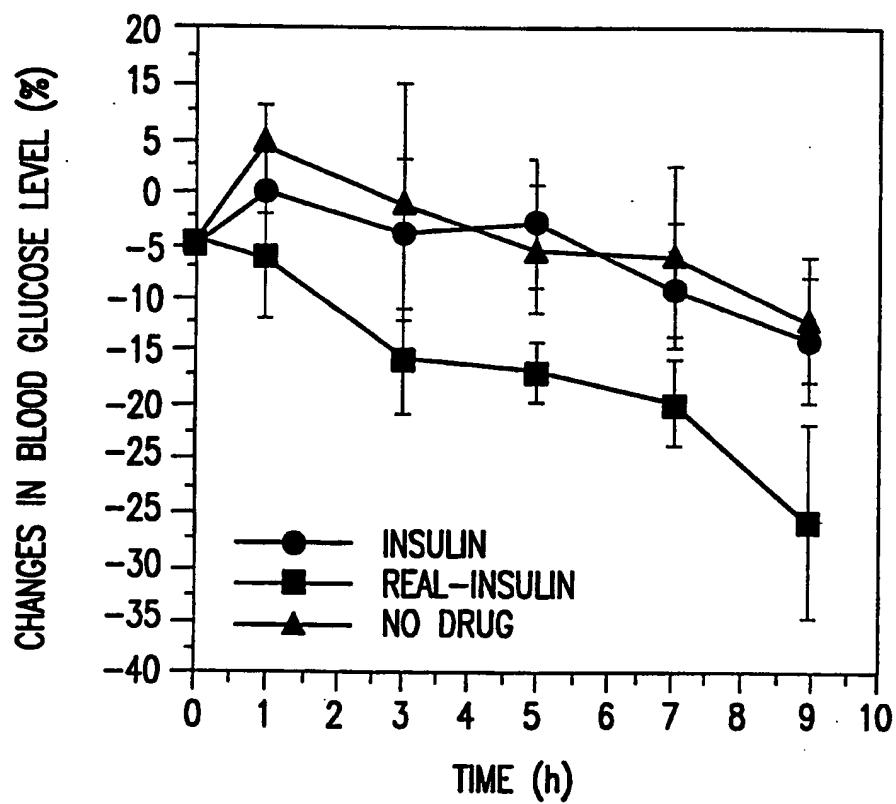


FIG.6

INTERNATIONAL SEARCH REPORT

Int. Application No.

PCT/US 99/29119

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07C323/60 C07K7/16 C07K14/62 C07C323/59 C07C323/54
C07D307/34 A61K31/195 A61K38/11 A61K38/28 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07C C07K C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 183 503 A (BEECHAM GROUP PLC) 4 June 1986 (1986-06-04) page 3, lines 29-34, in particular, line 32; examples 1-4; claims	1, 8, 9, 22, 25
A	M.M. KAYSER ET AL: CAN. J. CHEM., vol. 71, no. 7, 1993, pages 1010-1021, XP000877440 page 1018, table 5, entry 2	9
A	EP 0 495 265 A (AKZO NV) 22 July 1992 (1992-07-22) examples; claims	1, 9, 15, 23, 25
A	US 5 144 011 A (W.C. SHEN ET AL) 1 September 1992 (1992-09-01) the whole document	1, 9, 15, 23, 25
	-/-	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

30 March 2000

Date of mailing of the international search report

06/04/2000

Name and mailing address of the ISA

European Patent Office, P.B. 6818 Patentplan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3018

Authorized officer

Van Amsterdam, L

INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/US 99/29119

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>US 4 569 789 A (W.A. BLATTNER ET AL) 11 February 1986 (1986-02-11) the whole document</p>	<p>1,8,9, 15,21-25</p>

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 29119

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 22
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 22
is directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Appl. No.

PCT/US 99/29119

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 183503	A	04-06-1986	AU 5044585 A	17-07-1986
			DE 3586313 A	13-08-1992
			DK 552285 A	31-05-1986
			GR 852877 A	31-03-1986
			JP 2545339 B	16-10-1996
			JP 6279318 A	04-10-1994
			JP 2004135 C	20-12-1995
			JP 7017517 B	01-03-1995
			JP 61155333 A	15-07-1986
			PT 81581 A, B	01-12-1985
			US 4935465 A	19-06-1990
EP 495265	A	22-07-1992	AT 109476 T	15-08-1994
			AU 646121 B	10-02-1994
			AU 9009891 A	28-01-1993
			CA 2058595 A	01-07-1992
			DE 69103255 D	08-09-1994
			DE 69103255 T	08-12-1994
			DK 495265 T	02-01-1995
			ES 2061166 T	01-12-1994
			FI 916131 A	01-07-1992
			HU 60484 A	28-09-1992
			IE 65406 B	18-10-1995
			JP 4334377 A	20-11-1992
			NO 180417 B	06-01-1997
			NZ 241217 A	23-12-1993
			PT 99954 A, B	30-06-1993
			US 5306809 A	26-04-1994
			ZA 9110203 A	27-01-1993
US 5144011	A	01-09-1992	US 4631190 A	23-12-1986
US 4569789	A	11-02-1986	US 4542225 A	17-09-1985
			AT 72399 T	15-02-1992
			AT 135003 T	15-03-1996
			CA 1241949 A	13-09-1988
			DE 3585369 A	19-03-1992
			DE 3588090 D	11-04-1996
			DE 3588090 T	18-07-1996
			EP 0191772 A	27-08-1986
			EP 0408157 A	16-01-1991
			JP 1954589 C	28-07-1995
			JP 4356483 A	10-12-1992
			JP 6084372 B	26-10-1994
			JP 6102679 B	14-12-1994
			JP 62500100 T	16-01-1987
			WO 8601409 A	13-03-1986
			US 4618492 A	21-10-1986
			US 4764368 A	16-08-1988

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.